

## Viruria during acute Japanese encephalitis virus infection

ASHA MATHUR, NIVEDITA KHANNA, RAJESH KULSHRESHTHA,  
S.C. MAITRA\* AND U.C. CHATURVEDI

*Postgraduate Department of Microbiology, K.G. Medical College, Lucknow, and*

*\*E.M. Unit, Central Drug Research Institute, Lucknow 226 001, India*

Received for publication 1 September 1994

Accepted for publication 17 November 1994

**Summary.** In this study, viruria following Japanese encephalitis virus (JEV) infection in mice has been shown to appear earlier in pregnant than in normal mice with proteinuria and haematuria. This was related to the production of splenic macrophage derived neutrophil chemotactic factor (MDF) following JEV infection. Intravenous inoculation of MDF in mice resulted in leakage of cells, proteins and erythrocytes in the urine as a result of altered capillary permeability. The isolation of virus from kidney did not correlate with the shedding of virus in the urine. The histological examination of sections of kidneys showed no morphological damage; however, ultrastructural degenerative changes in the mesangial cells were observed following JEV infection. These data suggest that JEV-induced macrophage derived factor regulates the leakage of proteins, erythrocytes and cells into the urine.

**Keywords:** viruria, MDF, albuminuria, Japanese encephalitis virus

Japanese encephalitis virus (JEV) is a major human pathogen, particularly in South-East Asia, causing epidemic encephalitis each year (Umenai *et al.* 1985). Virus has been isolated following primary infection from brain tissue in fatal cases, CSF in non-fatal cases (Leake *et al.* 1986; Mathur *et al.* 1986; 1990) and rarely from blood (Kedarnath *et al.* 1984). The virus could also be detected by co-cultivation of peripheral blood mononuclear cells of latently JEV infected children (Sharma *et al.* 1991). During the acute stage of infection congestion, oedema and small haemorrhages with neuronal degeneration are found in brain. A number of pathological changes have also been noted in extraneural tissues, e.g. in myocardium, hepatic Kupffer cells, spleen, lymph nodes and focal haemorrhages in kidneys (Grascenkov 1964). The urinary tract symptoms, such as the presence of albumin and red blood cells in urine,

are also frequent during the acute state (Monath 1990). We have not come across any description of the isolation of JE virus from the urine following infection. Excretion of cytomegalovirus (Kajiwarra *et al.* 1993), BK and JC viruses (Sundsford *et al.* 1994), adenovirus (Stamm & Truck 1987) and infectious hepatitis virus (Conrad *et al.* 1964) in the urine of patients and Hantaan virus in rats (Lee *et al.* 1986) has been demonstrated.

During JEV infection the splenic macrophages secrete a macrophage derived neutrophil chemotactic factor (MDF) (Khanna *et al.* (1991). MDF is characterized as a 10 kDa polypeptide, with a variety of *in vivo* biological effects. It increases the capillary permeability (Khanna *et al.* 1994) resulting in the breakdown of the blood–brain barrier with leakage of plasma proteins, erythrocytes and cellular infiltration in brain parenchyma (Mathur *et al.* 1992). The present report describes the isolation of infectious virus from the urine of JEV infected mice which may be attributed to the action of MDF.

Correspondence: Professor Asha Mathur, Postgraduate Department of Microbiology, K.G. Medical College, Lucknow 226 003, India.

## Materials and methods

### *Virus and mice*

Japanese encephalitis virus (JEV) strain 78668A was isolated from human brain (Mathur *et al.* 1982). The JEV was propagated by intracerebral (i.c.) inoculation of mice and fifth-pass brains were stored at  $-70^{\circ}\text{C}$ . The infectivity titre of virus pool in suckling mice was  $10^{4.5}$  LD<sub>50</sub>/25  $\mu\text{l}$ . Inbred Swiss albino mice (6–7 weeks of age) or 8-day-old pregnant mice obtained from this Department were inoculated intraperitoneally with 0.3 ml of 10 LD<sub>50</sub> of JEV infected mouse brain suspension.

### *Virus isolation*

Kidneys from 7 to 11 JEV infected and control mice were collected at different intervals and cut lengthwise and imprint smears from cut surfaces were prepared on slides for indirect immunofluorescence. The other halves were used for virus isolation after chopping and washing with cold Hank's balanced salt solution (HBSS) until they were free of blood. A 10% (w/v) suspension was prepared in Eagle's minimum essential medium (MEM) with antibiotics. Blood by cardiac puncture and urine by aseptic aspiration from the bladder were collected individually at the same time. The urine pH was immediately adjusted to 7.2 and the urine was diluted 1:2 in HBSS. One-day-old infant mice were inoculated i.c. for virus isolation as described earlier (Mathur *et al.* 1986).

### *Indirect immunofluorescence (IF)*

The freshly collected urine was centrifuged at low speed (300g). The cell pellet was spotted on Teflon coated microscopic slides and the smears were air dried. The cell smears and kidney imprint smears were fixed in chilled acetone and examined by indirect immunofluorescence as described previously (Mathur *et al.* 1990). 1:100 dilution of JE virus specific monoclonal antibody designated 98.9.5i (kindly given by Dr E.A. Gould, Oxford, UK) was used. The smears were screened under a Dialux 20 Leitz fluorescent microscope.

### *Antibody estimation*

The haemagglutination inhibition (HAI) test was performed by the modified technique of Clarke and Casals (1958) as described earlier (Mathur *et al.* 1986). The reciprocal dilution of serum giving inhibition against

8 HA units of antigen is presented. Each HAI value represents means  $\pm$  s.e. from seven to nine mice.

### *Histopathological study of kidney*

Kidneys of JEV infected and control mice were removed at various intervals. Tissue pieces were fixed in 10% neutral buffered formalin. Paraffin blocks were made, sectioned and stained with haematoxylin and eosin. Sections were coded, examined and expressed as + to + + + according to the increasing degree of changes in glomeruli and tubules.

### *Electron microscopy*

Kidney tissue pieces of approximately  $1 \times 1$  mm size were fixed at  $4^{\circ}\text{C}$  in 3.5% glutaraldehyde. After 30 minutes pieces were washed with sodium cacodylate buffer and post-fixed in 1% cacodylate buffered osmium tetroxide at  $4^{\circ}\text{C}$  for 1 h followed by three washings in cacodylate buffer. Following dehydration in acetone series the tissues were embedded in Durcupan mixture and sectioned. Sections were stained with 1% aqueous uranyl acetate and 0.2% lead citrate and examined in a Philips electron microscope (410LS).

### *Preparation of macrophage derived factor (MDF)*

The MDF was prepared from splenic macrophages of JEV primed Swiss albino mice and purified as described earlier (Khanna *et al.* 1991). Briefly, mice were given 0.3 ml of 10 LD<sub>50</sub> of JEV i.p. On day 7 post infection the spleens were collected and the cells were cultured in MEM-HEPES with antibiotics for 2 h at  $37^{\circ}\text{C}$  in the presence of 5% CO<sub>2</sub>. The adherent cells were washed thrice with phosphate buffered saline (PBS) and incubated at  $37^{\circ}\text{C}$  in normal saline for 24 h. The macrophage derived factor was collected and purified on Sephacryl S-200 column (Pharmacia, Sweden). The neutrophil chemotactic activity was assayed by modified Boyden Chamber technique using 5  $\mu\text{m}$  pore size nitrocellulose filter (Khanna *et al.* 1991).

### *Assay of albumin in mice*

The leakage of albumin in urine of mice was measured by the stick test using Ames plastic strips (Miles India Ltd, Baroda, India) (McMurray 1988). The urethra was tied and mice were inoculated intravenously with 200  $\mu\text{g}$  of macrophage derived neutrophil chemotactic factor or with normal mouse macrophage supernatant. The viscera was opened 1 h later and urine was collected

**Table 1.** Isolation of JEV from urine and kidney in pregnant and normal mice following JEV infection

Days after infection	Pregnant		Non-pregnant	
	Kidney	Urine	Kidney	Urine
1	0/7	0/7	0/7	0/7
3	0/7	0/7	0/7	0/7
5	2 ± 0.24/11	2 ± 0.12/11	0/11	0/11
7	4 ± 1.5/11*	3 ± 0.34/11	3 ± 0.99/11*	0/11
9	4 ± 1.1/11**	2 ± 0.23/11	1 ± 0.39/11**	2 ± 0.32/11*
Control	0/7	0/7	0/7	0/7

\*  $P > 0.05$ ; \*\*  $P < 0.01$ .

Number of mice with virus/number tested per experiment. Each value represents means ± s.e. of three different experiments.

aseptically from the bladder. The absorbant area of the strip was dipped in urine samples and the colour produced after 30 s was compared with the test chart and expressed as mg/dl. The sensitivity of the test was 15–30 mg albumin/dl.

## Results

### Detection of JEV

Groups of normal and 8-day-pregnant mice were inoculated i.p. with 10 LD<sub>50</sub> of JEV. Table 1 shows the rate of virus recovery from the kidney and urine of individual animals from both groups of mice. The virus was first isolated from kidney on day 5 from pregnant (2 ± 0.54/11) and on day 7 from non-pregnant (3 ± 0.99/11) groups of mice after infection. The virus recovery was significantly higher ( $P < 0.01$ ) in pregnant mice (4 ± 1.1/11) as compared to the non-pregnant mice (1 ± 0.39/11) on day 9 p.i. No virus was isolated from control groups of mice. To determine the distribution of antigen positive cells in kidney, the imprint smears obtained from JEV infected pregnant and non-pregnant mice were screened on different days. JEV specific immunofluorescence positive cells were seen distributed in numerous foci. The tubular epithelium showed no antigen positive cells. Blood showed presence of virus on day 1 in non-pregnant and on days 1 and 2 in pregnant groups of mice.

An attempt was made to isolate infectious virus from urine of JEV infected mice. Viruria was detected from day 5 to 9 in the pregnant group and the infectivity titre ranged from 10<sup>-1</sup> to 10<sup>-2.1</sup>/25 µl, while in the non-pregnant group the virus was recovered from urine on day 9 (2 ± 0.32/11) p.i. only (the virus titre was 10<sup>-1.2</sup>/25 µl). No virus was recovered from urine of

**Table 2.** JEV titres in the urine of pregnant and non-pregnant groups of mice on different days

Days p.i.	Pregnant	Non-pregnant
1	—	—
3	—	—
5	10 <sup>-1 ± 0.08</sup>	—
7	10 <sup>-1.8 ± 0.13</sup>	—
9	10 <sup>-2.1 ± 0.25**</sup>	10 <sup>-1.2 ± 0.15**</sup>

\*\*  $P < 0.005$ .

Each value represents mean ± s.e. of three experiments.

control mice receiving normal mouse brain suspension (Table 2). The indirect immunofluorescence of urinary exfoliated cells in the pregnant group using anti-JEV monoclonal antibodies revealed the presence of 3 ± 0.83% of immunofluorescent positive cells on day 5 p.i. which gradually increased and reached a maximum on day 9 p.i. (12 ± 2%).

### Antibody response in mice

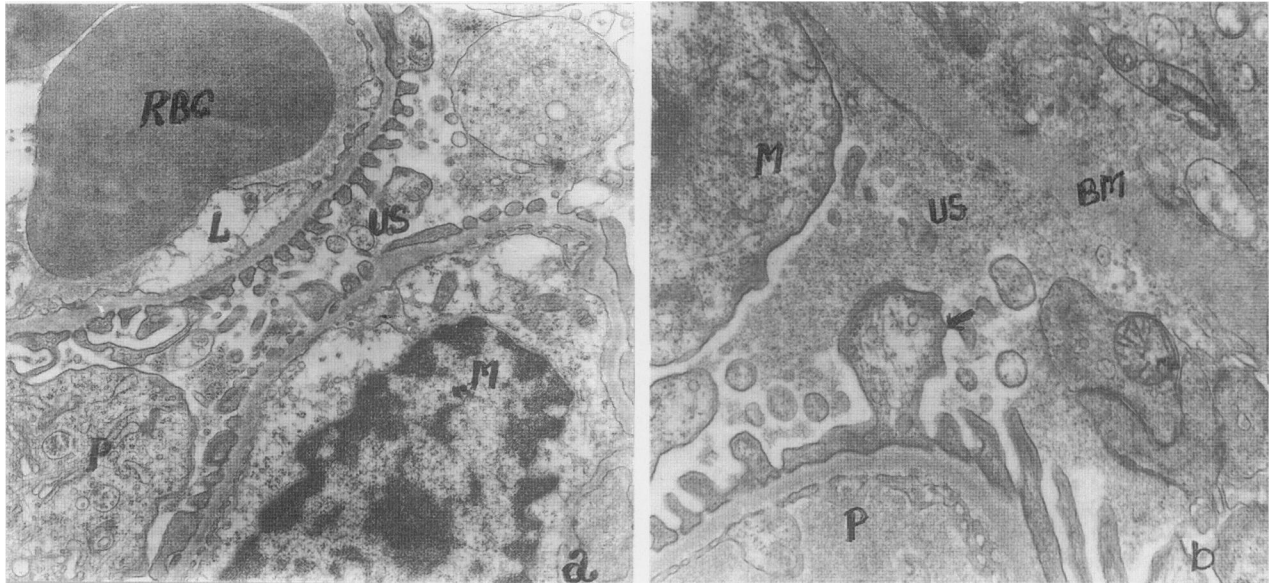
The HAI antibodies were detected in groups of mice before and after JEV inoculation. All mice were negative (< 1:8) for JEV antibodies before virus was given. JEV infection was followed by a more than fourfold rise in HAI antibody titre, an indication of active infection in both groups of mice. The mean JEV HAI antibody titres were 1:46 ± 5 in the pregnant and 1:40 ± 2 in the non-pregnant groups of mice on day 9 p.i.

### Relation between renal and urinary virus isolation

The virus was isolated from the renal tissue of 10 pregnant and 4 non-pregnant mice from day 5 to 9 following infection while excretion of the virus from urine was demonstrated in only 7 out of 47 pregnant and 2 out of 47 non-pregnant mice. Three of the pregnant mice which had virus in urine did not show the virus in renal tissue. Virus was isolated on day 9 p.i. from kidney of only one non-pregnant mouse while viruria was detected in two mice.

### Histopathology

Light microscopic examination of kidney in JEV infected pregnant and non-pregnant mice revealed mild hypercellularity. Infiltrating cells were predominantly lymphocytes with few macrophages. Small haemorrhagic areas were noticed. Tubules showed no change.

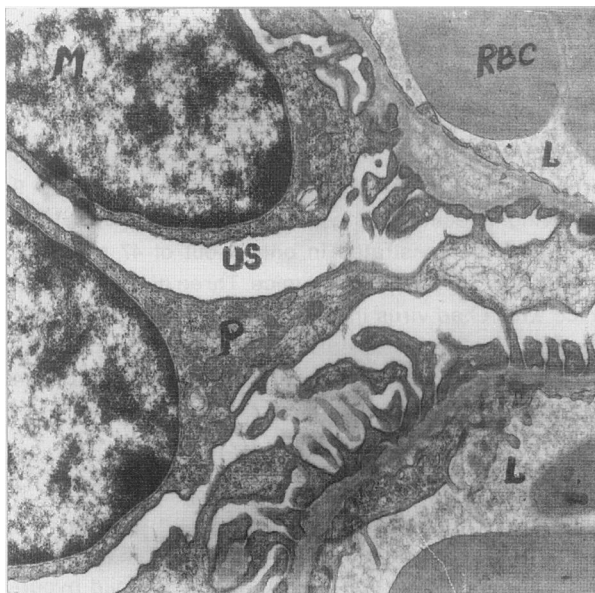


**Figure 1.** a, Electron micrograph of mouse kidney showing hypertrophic changes in podocyte and degenerative changes in mesangial cell on day 7 following JEV infection.  $\times 12000$ . b, Electron micrograph showing cyst formation by foot process ( $\leftarrow$ ) and flocculent material in urinary space.  $\times 24000$ .

#### *Ultrastructural changes in JEV infected mouse kidney*

On day 7 post infection the lumen (L) of the glomerular capillary showed moderately electron dense material and erythrocytes (Figure 1a). The podocytes (P) showed varying degree of hypertrophic changes, as the

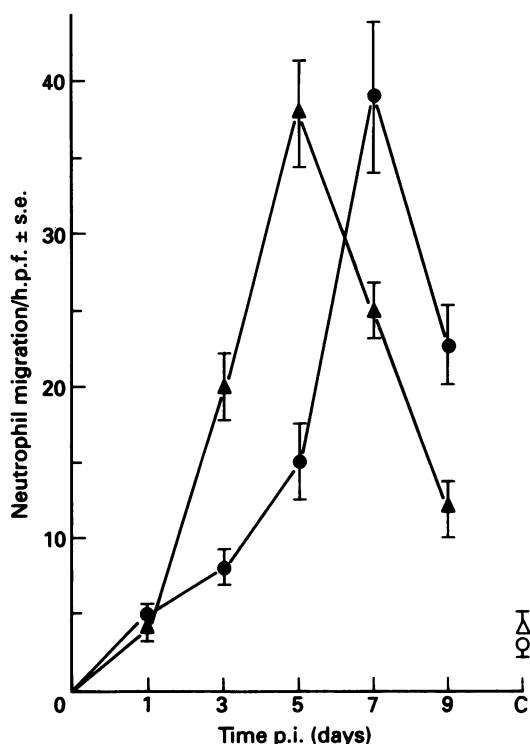
cytoplasm contained elaborate Golgi complex, scattered endoplasmic reticulum and microbodies. There was also seen some cyst ( $\leftarrow$ ) formation by foot processes (Figure 1b), while controls showed no such formations (Figure 2). Occasional subepithelial deposits on basement membrane (BM) were present. The urinary space (US) was full of flocculent materials including multi-vesicular bodies (Figure 1a and b), in contrast to the controls where the urinary space was clear (Figure 2). Degeneration changes in mesangial cells (M) were evidenced by their pyknotic nucleus and vacuolated cytoplasm (Figure 1a).



**Figure 2.** Electron micrograph of uninfected mouse kidney.  $\times 12000$ . L, Lumen; US, urinary space; P, podocyte; M, mesangial cell; BM, basement membrane; RBC, red blood cell.

#### *Production of neutrophil chemotactic activity by splenic cells of JEV primed pregnant mice*

The present experiment was carried out to ascertain the day of appearance of the neutrophil chemotactic response by splenic macrophage supernatant of JEV primed pregnant mice and to compare it with time of production of MDF by splenic macrophages of JEV primed non-pregnant mice. The findings presented in Figure 3 show that in JEV primed non-pregnant mice maximum production of MDF occurred on day 7 p.i., whereas in JEV primed pregnant neutrophil chemotactic activity appeared early and the peak activity was observed on day 5. The control supernatants did not show chemotactic activity.



**Figure 3.** Production of macrophage derived factor in the spleens of ▲, pregnant and ●, non-pregnant mice during JEV infection. C, control mice were given normal mouse brain suspension in △, pregnant and ○, non-pregnant groups of mice. Each point represents mean  $\pm$  s.e. of 5 duplicate experiments.

#### Protein leakage and microscopic examination of urine

Groups of MDF inoculated and control mice were examined for protein leakage in urine using Ames plastic strips. The urine collected at 1 h after MDF inoculation induced a colour change from yellow to bluish-green corresponding to a protein concentration of 30 mg/dl. The urine from control mice which were given 100  $\mu$ l of normal mouse macrophage supernatant did not cause any colour change on the strips, thus indicating a negative response.

The microscopic examination of urine sediments showed a significant haematuria after MDF inoculation ( $15 \pm 1$  RBC/high power field). In addition a few white cells and epithelial cells were also seen showing an inflammatory response after MDF inoculation. No such cellular infiltration was observed in urine deposits of control mice.

#### Discussion

In this report we present evidence that JEV infection in

mice could lead to transient changes in kidney and shedding of virus in urine. The virus excretion in pregnant mice occurred four days earlier than that in non-pregnant mice. There was no correlation between simultaneous virus isolation from kidney and urine. The excretion of virus in urine seems to be related to the day of maximum production of macrophage derived neutrophil chemotactic factor. We observed that i.v. injection of purified MDF in mice causes albuminuria, haematuria and shedding of cells in urine indicating that MDF could regulate shedding of virus in urine. Viruria has not been demonstrated earlier in JEV infection (Monath 1990).

Our results demonstrate viraemia for the first two days after JEV infection and then the virus disseminates to different tissues where further multiplication occurs (Mathur *et al.* 1986). Most of the arboviruses are small and cleared by the reticuloendothelial system; therefore, a large dose is necessary to reach the CNS (Johnson 1987). We isolated infectious virus from kidney following JEV infection which appeared early in pregnant as compared to non-pregnant mice. This could be because of the differences in renal blood flow between pregnant and non-pregnant animals resulting in early virus replication in the organs of infected pregnant mice.

Interestingly we have detected early production of macrophage derived factor in pregnant as compared to non-pregnant mice during JEV infection. This coincides with virus excretion in the urine though at this time there occurs no morphological damage in renal parenchyma. This raises the possibility that MDF regulates the virus shedding in urine as it enhances the capillary permeability. The localization of JEV antigen on endothelial capillaries and precapillaries of parenchymatous organs (Grascenkov 1964) and brain capillaries (Johnson *et al.* 1985) has been observed which may or may not precede further infection. CMV infection in humans can also result in chronic viruria, though the source of infection is not clear. It is suggested that steroids, peptide hormones and genetic factors might contribute to its pathogenesis (Knox *et al.* 1979).

We have previously demonstrated that MDF induced an increase in capillary permeability resulting in the leakage of plasma proteins, radiolabelled erythrocytes and cells into the tissues or at the site of injection (Khanna *et al.* 1994; Mathur *et al.* 1992). In the present study we found that mice inoculated with MDF showed albuminuria, microscopic haematuria and a fair number of cells in the urine. These findings are consistent with albuminuria and haematuria and sterile pyuria observed in human JE patients (Monath 1990). The present findings are supported by electron microscopic examination which showed mild inflammatory and

tissue degenerative changes in renal parenchyma and urinary spaces showed flocculent material on day 7 after JEV infection.

MDF produced during JEV infection is an important proinflammatory factor which induces accumulation of neutrophils and modulates their activation via a protein kinase C dependent mechanism (Khanna *et al.* 1993), resulting in augmentation of inflammatory response. The signal transduction has been shown to be initiated with a rise in cytosolic  $Ca^{2+}$  in neutrophils, the requirement for which is obligatory for receptor mediated cell stimulation (Srivastav *et al.* 1994). On the basis of the positioning of the cysteine residues in a conserved motif, the chemokines have been divided into two subfamilies: the CXC, neutrophil chemokines are IL-8, melanoma growth stimulating activity and platelet factor 4, while CC chemokines include RANTES, monocyte chemotactic protein-1 and macrophage inflammatory protein-1 which attract monocytes (Horuk 1994). The complete biochemical characterization of MDF on the basis of molecular structure is yet to be established.

Japanese encephalitis virus, a member of the family Flaviviridae, is the most common cause of encephalitis worldwide with high case fatality rate (Johnson 1987). However, the pathogenesis of infection is not very clear. We suggest that MDF should not be used exclusively as a neutrophil chemotactic factor since its major activity is vascular alteration leading to disease production. Further work is in progress to utilize the adverse effect of this cytokine to study JEV induced pathology by active immunization with MDF.

## Acknowledgements

The authors thank Dr K.L. Arora, who initiated the virus isolation studies. This study was carried out with the financial assistance of the Department of Science and Technology, Government of India, New Delhi.

## References

- CLARKE D. H. & CASALS J. (1958) Techniques of haemagglutination and haemagglutination inhibition with arthropod-borne viruses. *Am. J. Trop. Med. Hyg.* **7**, 561–573.
- CONRAD M.E. Jr., SCHWARTZ F.O. & YOUNG A.A. (1964) Infectious hepatitis-A generalized disease. A study of renal, gastrointestinal and hematologic abnormalities. *Am. J. Med.* **37**, 789–801.
- GRASCENKOV, N.I. (1964) Japanese encephalitis in the USSR. *Bull. WHO* **30**, 161–172.
- HORUK, R. (1994) The IL-8 receptor family: from chemokines to malaria. *Immunol. Today* **15**, 169–174.
- JOHNSON, R.T. (1987) The pathogenesis of acute viral encephalitis and post infectious encephalomyelitis. *J. Infect. Dis.* **155**, 359–364.
- JOHNSON, R.T., BURKE, D.S., ELWELL, H., LEAKE, C.J., NISALAK, A., HOKE, C.H., & LORSOMRUEDEE, W. (1985) Japanese encephalitis: Immunocytochemical studies of viral antigen and inflammatory cells in fatal cases. *Ann. Neurol.* **18**, 567–573.
- KAJIWARA, M., YAMAGUCHI, Y., HIRAI, K. & YATA, J. (1993) Increased urinary excretion of human cytomegalovirus in children with malignancy: detection by polymerase chain reaction. *Acta Paediatr. Jpn.* **35**, 387–393.
- KEDARNATH, N., PRASAD, S.R., DANDAWATE, C.N., KOSHY, A.A., GEORGE, S. & GHOSH, S.N. (1984) Isolation of Japanese encephalitis and West Nile viruses from peripheral blood of encephalitis cases. *Indian J. Med. Res.* **79**, 1–7.
- KHANNA, N., AGNIHOTRI, M., MATHUR, A. & CHATURVEDI, U.C. (1991) Neutrophil chemotactic factor produced by Japanese encephalitis virus stimulated macrophages. *Clin. Exp. Immunol.* **86**, 299–303.
- KHANNA, N., MATHUR, A., CHATURVEDI, U.C. (1994) Regulation of vascular permeability by macrophage derived chemotactic factor produced in Japanese encephalitis. *Immunol. Cell. Biol.* **72**, 200–204.
- KHANNA, N., SRIVASTAV, S., MATHUR, A. & CHATURVEDI, U.C. (1993) Stimulation of neutrophil respiratory burst and degranulation by Japanese encephalitis virus-induced macrophage-derived factor. *Int. J. Exp. Path.* **74**, 339–345.
- KNOX, G.E., PASS, R.F., REYNOLDS, D.W., STAGNO, S. & ALFORD, C.A. (1979) Comparative prevalence of subclinical cytomegalovirus and herpes simplex virus infections in the genital and urinary tracts of low income, urban women. *J. Infect. Dis.* **140**, 419–422.
- LEAKE, C.J., BURKE, D.S., NISALAK, A. & HOKME, C.H. (1986) Isolation of Japanese encephalitis virus from clinical specimens using a continuous mosquito cell line. *Am. J. Trop. Med. Hyg.* **35**, 1045–1050.
- LEE, P.W., YANAGIHARA, GIBBS, C.J. Jr., GAJDUSEK, D.C. (1986) Pathogenesis of experimental Hantaan virus infection in laboratory rats. *Arch. Virol.* **88**, 57–66.
- MATHUR, A., ARORA, K.L., CHATURVEDI, U.C. (1986) Persistence, latency and reactivation of JEV in mice. *J. Gen. Virol.* **67**, 381–385.
- MATHUR, A., CHATURVEDI, U.C., TANDON, H.O., AGARWAL, A.K., MATHUR, G.P., NAG, D., PRASAD, A. & MITTAL, V.P. (1982) Japanese encephalitis in Uttar Pradesh, India, during 1978. *Indian J. Med. Res.* **75**, 161–169.
- MATHUR, A., KHANNA, N. & CHATURVEDI, U.C. (1992) Breakdown of blood-brain barrier by virus induced cytokine during Japanese encephalitis virus infection. *Int. J. Exp. Path.* **73**, 603–611.
- MATHUR, A., KUMAR, R., SHARMA, S., KULSHRESHTHA, R., KUMAR, A. & CHATURVEDI, U.C. (1990) Rapid diagnosis of Japanese encephalitis by immunofluorescence examination of cerebrospinal fluid. *Indian J. Med. Res.* **91**, 1–4.
- McMURRAY J.R. (1988) Proteins in urine, cerebrospinal fluid and other fluids. In *Varley's Practical Clinical Biochemistry*, 6th edition. Ed. A.H. Gowenlock p. 438.
- MONATH T.P. (1990) Flaviviruses. In *Fields Virology*, Volume 1. Eds B.N. Fields & D.M. Knipe. New York: Raven Press, pp. 763–814.
- SHARMA, S., MATHUR, A., PRAKASH V., KULSHRESHTHA, R., KUMAR, R. & CHATURVEDI, U.C. (1991) Japanese encephalitis virus latency in peripheral blood lymphocytes and recurrence of infection in children. *Clin. Exp. Immunol.* **85**, 85–89.
- SRIVASTAV S., KHANNA N., MATHUR A. & CHATURVEDI U.C. (1994) Role of calcium in neutrophil activation by Japanese encephalitis

- virus-induced macrophage-derived factor. *Acta Virol.* **34**, 111–116.
- STAMM W.E. & TRUCK M. (1987) Urinary tract infections, pyelonephritis and related conditions. In *Harrison's Principles of Internal Medicine*. Eleventh edition. K.J. Isselbacher, R.G. Petersdorf, J.D. Wilson & A.S. Fauci, New York: McGraw Hill Book Company. p. 1189.
- SUNDSFJORD A., FLAEGESTAD T., FLO R., SPEIN A.R., PEDERSON M., PERMIN, H. JULSRUD J & TRAAVIK T. (1994) B.K. and JC viruses in human immunodeficiency virus Type-1 infected persons: prevalence, excretion, viraemia and viral regulatory regions. *J. Infect. Dis.* **169**, 485–490.
- UMENAI T., KRZYSKO R., BETIMEROV T.A. & ASSAD F.A. (1985). Japanese encephalitis: current world wide status. *Bull. WHO* **63**, 625–631.
- UTZ J.P., HOUK, V.N. ALLING D.W. (1964) Clinical and laboratory studies of mumps IV. Viruria of abnormal renal function. *New Engl. J. Med.* **270**, 1283–1286.